

THERAPEUTIC METHODS COMPRISING USE OF A NEUREGULIN

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods for treatment of certain neurological-related injuries and disorders comprising use of a neuregulin, or a fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or neuregulin fragment or derivative.

2. Background

Nerve cell death (degeneration) can cause potentially devastating and irreversible effects for an individual and may occur e.g. as a result of stroke, heart attack or other brain or spinal chord ischemia or trauma. Additionally, neurodegenerative disorders involve nerve cell death (degeneration) such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome and Korsakoff's disease.

Therapies have been investigated to treat nerve cell degeneration and related disorders, e.g., by limiting the extent of nerve cell death that may otherwise occur to an individual as well as promoting repair, remodeling and reprogramming after stroke or other neuronal injury. See, e.g., F. Seil, *Curr Opin Neuro*, 10:49-51 (1997); N. L. Reddy et al., *J Med Chem*, 37:260-267 (1994); and WO 95/20950.

Certain growth factors have been reported to exhibit neuroprotective properties. In particular, nerve growth factor (NGF) has been evaluated in certain neuroprotective models. See, for example, G. Sinson et al., *J Neurosurg*, 86(3):511-518 (1997); and G. Sinson et al., *J Neurochem*, 65(5):2209-2216 (1995). Osteogenic protein-1 (OP-1) has been evaluated in a rat model of cerebral hypoxia/ischemia for neuroprotective activity. G. Perides, *Neurosci Lett*, 187(1):21-24 (1995). Glial cell line-derived neurotrophic factor (GDNF) was reported to exhibit trophic activity on certain populations of central neurons. Y. Wang et al., *J Neurosci*, 17(11):4341-4348 (1997). Small molecules also have been investigated as neuroprotective agents, such

as MK-801. See B. Meldrum, *Cereb Brain Metab Rev*, 2:27-57 (1990); D. Choi, *Cereb Brain Metab Rev*, 2:27-57 (1990).

However, no effective pharmacotherapies are in regular clinical use for ischemia-induced brain injury or other such injuries and disorders. See, for example,
5 Y. Wang et al., *supra*; G. Sinson et al., *J Neurochem*, 65(5):2209 (1995).

It thus would be highly desirable to have new neuroprotective agents, particularly agents to limit the extent or otherwise treat nerve cell death (degeneration) that occur with stroke, heart attack or brain or spinal cord trauma, or to treat
10 Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome and Korsakoff's disease. It also would be desirable to have agents that promote repair, remodeling or reprogramming after stroke or other neuronal injury.

SUMMARY OF THE INVENTION

15 The present invention provides methods for treatment and/or prophylaxis of certain neurological-related disorders, particularly treatment or prophylaxis of the effects of stroke, brain or spinal cord injury or ischemia, heart attack, optic nerve and retinal injury and ischemia and other acute-type conditions disclosed herein as well as chronic-type conditions, specifically epilepsy, Alzheimer's disease, Parkinson's
20 disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Down's Syndrome, Korsakoff's disease, cerebral palsy and/or age-dependent dementia. Methods of the invention also include therapies for promoting repair, remodeling or reprogramming after stroke or other neuronal injury.

The methods of the invention comprise administration of an effective amount
25 of neuregulin, or fragment or derivative of a neuregulin, or a nucleic acid encoding a neuregulin or a neuregulin fragment or derivative (i.e. gene therapy), to a patient suffering from or susceptible to such conditions.

Neuregulins are members of the epidermal growth factor (EGF) superfamily and include glial growth factor (GGF), acetylcholine receptor-inducing activity
30 (ARIA), neu differentiation factor (NDF) and heregulins (HRF). See D. E. Wen et al., *Cell*, 69:559-572 (1992); W.E. Holmes et al., *Science*, 256:1205-1210 (1992); M.A. Marchionni et al., *Nature*, 362:312-318 (1993); and D.L. Falls, *Cell*, 72:801-815

(1993). A variety of neuregulins and fragments and derivatives thereof can be employed in the methods of the invention. For example, suitable agents have been disclosed in U.S. Patent 5,530,109 and PCT/US93/07491. Neuregulins also have been reported in U.S. Patent 5,367,060. Preferred neuregulins include regions shown in FIGS. 1-2 (SEQ ID NOS. 2 and 4), also known as the E sequence. Preferred neuregulins or fragments or derivatives also include those that contain the C, C/D or C/D' sequences as shown in Figures 7, 8 and 9 respectively of the drawings, or those neuregulins or fragments or derivatives that have substantial homology to the peptide sequences shown in Figures 7, 8 or 9, e.g. at least about 70 percent homology, or at least about 80 percent homology, or more preferably at least about 90 or 95 percent homology to the peptide sequences shown in Figures 7, 8 or 9. Preferred nucleic acids and fragments and derivatives for use in the methods of the invention include those nucleic acids that include one or more nucleic acids sequences shown in Figures 7, 8 and 9 of the drawings, or those nucleic acids that have substantial homology to the nucleic acid sequences shown in Figures 7, 8 or 9, e.g. at least about 70, 80, 90 or 95 percent homology to the nucleic acid sequences shown in Figures 7, 8 or 9. A particularly preferred neuregulin is encoded by DNA obtainable from the clone pGGF2HBS11 (ATCC Deposit No. 75347). Also preferred are neuregulins encoded by DNA obtainable from GGF2BPP5, GGF2BPP2 and GGF2BPP4.

Typical patients that may be treated in accordance with the methods of the invention are persons suffering from brain or spinal cord trauma or ischemia, stroke, heart attack, hypoxia, hypoglycemia, post-surgical neurological deficits, decreased blood flow or nutrient supply to retinal tissue or optic nerve, retinal trauma or ischemia or optic nerve injury. Patients suffering from chronic-type conditions also may be treated in accordance with the invention, specifically subjects suffering from or susceptible to epilepsy, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Down's Syndrome, Korsakoff's disease, cerebral palsy and/or age-dependent dementia.

Also, as discussed above, a neuregulin or fragment or derivative thereof or nucleic acid encoding same, may be administered to promote repair, remodeling or reprogramming to a subject that has suffered stroke or other neuronal injury such as traumatic brain or spinal cord injury. In such cases, the therapeutic agent may be

suitably administered to the subject over an extended period following the injury, e.g. at least about 1, 2, 3, 4, 6, 8, 12 or 16 weeks following the injury.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

5 FIG. 1 shows a nucleotide sequence (SEQ ID NO:1) encoding a preferred neuregulin region (E segment of human GGF) and the amino acid sequence (SEQ ID NO:2) of that preferred region.

10 FIG. 2 shows a nucleotide sequence (SEQ ID NO:3) encoding a preferred neuregulin region (E segment of bovine GGF) and the amino acid sequence (SEQ ID NO:4) of that preferred region.

15 FIG. 3 shows nucleotide sequences (SEQ ID NOS:6-7) encoding further neuregulin regions (B segment of human and bovine GGF) and amino acid sequences (SEQ ID NOS:5 and 8) of those regions. Line 1 is the predicted amino acid sequence of bovine B segment, line 2 is a nucleotide sequence of bovine B segment, line 3 is a nucleotide sequence of human B segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human B segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

20 FIG. 4 shows nucleotide sequences (SEQ ID NOS:10-11) encoding further neuregulin regions (A segment of human and bovine GGF) and amino acid sequences (SEQ ID NOS:9 and 12) of those regions. Line 1 is the predicted amino acid sequence of bovine A segment, line 2 is a nucleotide sequence of bovine A segment, line 3 is a nucleotide sequence of human A segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human A segment shown where it differs from the bovine sequence set forth in line 1
25 of the figure.

 FIG. 5 shows a nucleotide sequence (SEQ ID NO:13) encoding a further neuregulin region (A' segment of bovine GGF) and the predicted amino acid sequence (SEQ ID NO:14) of that region.

30 FIG. 6 shows nucleotide sequences (SEQ ID NOS:16-17) encoding further neuregulin regions (G segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:15 and 18) of that region. Line 1 is the predicted amino acid sequence of bovine G segment, line 2 is a nucleotide sequence of bovine G segment, line 3 is a

nucleotide sequence of human G segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human G segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 7 shows nucleotide sequences (SEQ ID NOS:20-21) encoding further
5 neuregulin regions (C segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:19 and 22) of those regions. Line 1 is the predicted amino acid sequence of bovine C segment, line 2 is a nucleotide sequence of bovine C segment, line 3 is a nucleotide sequence of human C segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of
10 human C segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 8 shows nucleotide sequences (SEQ ID NOS:24-25) encoding further
neuregulin regions (C/D segment of human and bovine GGF) and amino acid
sequences (SEQ ID NOS:23 and 26) of those regions. Line 1 is the predicted amino
15 acid sequence of bovine C/D segment, line 2 is a nucleotide sequence of bovine C/D segment, line 3 is a nucleotide sequence of human C/D segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human C/D segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 9 shows nucleotide sequences (SEQ ID NOS:28-29) encoding a further
neuregulin region (C/D' segment of the human and bovine GGF) and the amino acid
sequence (SEQ ID NO:27) of that region. Line 1 is the predicted amino acid sequence
of the C/D' segment, line 2 is a nucleotide sequence of bovine C/D' segment and line
3 is a nucleotide sequence of human C/D' segment (nucleotide base matches are
25 indicated with a vertical line).

FIG. 10 shows nucleotide sequences (SEQ ID NOS:31-32) encoding a further
neuregulin region (D segment of the human and bovine GGF) and the amino acid
sequence (SEQ ID NO:30) of that region. Line 1 is the predicted amino acid sequence
of the D segment, line 2 is a nucleotide sequence of bovine D segment and line 3 is a
30 nucleotide sequence of human D segment (nucleotide base matches are indicated with a vertical line).

FIG. 11 shows nucleotide sequence (SEQ ID NO:34) encoding a further neuregulin region (D' segment of bovine GGF) and the amino acid sequence (SEQ ID NO:33) of that region.

FIGS. 12A-12B show nucleotide sequences (SEQ ID NOS:36-37) encoding further neuregulin regions (H segment of human and bovine GGF) and amino acid sequences (SEQ ID NO:35 and 38) of that region. Line 1 is the predicted amino acid sequence of bovine H segment, line 2 is a nucleotide sequence of bovine H segment, line 3 is a nucleotide sequence of human H segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human H segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 13 shows a nucleotide sequence (SEQ ID NO:40) encoding a further neuregulin region (K segment of bovine GGF) and the amino acid sequence (SEQ ID NO:39) of that region.

FIGS. 14A-14C show nucleotide sequences (SEQ ID NOS:42-43) encoding a further neuregulin region (L segment of bovine and human GGF) and amino acid sequences (SEQ ID NO:41 and 44) of that region. Line 1 is the predicted amino acid sequence of bovine L segment, line 2 is a nucleotide sequence of bovine L segment, line 3 is a nucleotide sequence of human L segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human L segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIG. 15 shows nucleotide sequences (SEQ ID NOS:46-47) encoding further neuregulin regions (F segment of bovine and human GGF) and amino acid sequences (SEQ ID NOS:45 and 48) of that region. Line 1 is the predicted amino acid sequence of bovine F segment, line 2 is a nucleotide sequence of bovine F segment, line 3 is a nucleotide sequence of human F segment (nucleotide base matches are indicated with a vertical line), and line 4 is the predicted amino acid sequence of human F segment shown where it differs from the bovine sequence set forth in line 1 of the figure.

FIGS. 16A-16C show the nucleotide sequence (SEQ ID NO:49) and deduced amino acid sequence (SEQ ID NO:50) of GGF2BPP4.

FIGS. 17A-17B show the nucleotide sequence (SEQ ID NO:51) and deduced amino acid sequence (SEQ ID NO:52) of GGF2BPP2.

FIGS. 18A-18B show the nucleotide sequence (SEQ ID NO:53) and deduced amino acid sequence (SEQ ID NO:54) of GGF2BPP5.

5 DETAILED DESCRIPTION OF THE INVENTION

As discussed above, preferred neuregulins for use in the therapeutic methods of the present invention include those disclosed in U.S. Patent 5,530,109 and PCT/US93/07491, incorporated herein by reference. Particularly preferred neuregulins comprise an amino acid sequence of the following formula:

10 WYBAZCX

wherein WYBAZCX is composed of amino acid sequences that include one or more sequences shown in FIGS. 1 through 15 (which includes SEQ ID NOS:2, 4, 5, 8, 9, 12, 14, 15, 18, 19, 22, 23, 26, 27, 30, 33, 35, 38, 39, 41, 44, 45 and 48), wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the

15 polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprise a polypeptide segment selected from the group consisting of C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/d D'H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL and C/D C/D' D' HKL, and preferably that either

- 20 a) at least one of F, Y, B, A, Z, C or X is of bovine origin; or
- b) Y comprises the polypeptide segment E; or
- c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D'H, C/D C/D' D HL, C/D C/D' D' HKL, C/D'H, C/D C/D' H or C/D C/D' HL.

Particularly preferred neuregulins also include those polypeptides that include the segments FB polypeptides that include the segments FBA' (i.e. the groups F, B and A' as defined herein including in the drawings); polypeptides that include the segments EBA (i.e. the groups E, B and A as defined herein including in the drawings); polypeptides that include the segments EBA' (i.e. the groups E, B and A' as defined herein including in the drawings); A (i.e. the group A as defined herein

including in the drawings); polypeptides that include the segments FEBA (i.e. the groups F, E, B and A as defined herein including in the drawings); polypeptides that include the segments FBA' (i.e. the groups F, B and A' as defined herein including in the drawings); and polypeptides that include the segments FEBA' (i.e. the groups F, E, B and A' as defined herein including in the drawings).

Also preferred are nucleic acids that code for the above preferred polypeptides.

A "fragment" or "derivative" of a neuregulin refers to herein 1) a peptide in which one or more amino acid residues are with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) a peptide in which one or more of the amino acid residues includes a substituent group, or (iii) a peptide in which the mature protein is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol). Thus, a fragment or derivative for use in accordance with the methods of the invention includes a proprotein, which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide fragments and derivatives of the invention are of a sufficient length to uniquely identify a region of a neuregulin. Neuregulin fragments thus preferably comprise at least 8 amino acids, usually at least about 12 amino acids, more usually at least about 15 amino acids, still more typically at least about 30 amino acids, even more typically at least about 50 or 70 amino acids. Preferred fragments or derivatives for use in the methods of the invention include those that have at least about 70 percent homology (sequence identity) to any of the preferred sequences mentioned above, more preferably about 80 percent or more homology to any of the preferred sequences mentioned above, still more preferably about 85 to 90 percent or more homology to any of the preferred sequences mentioned above. Sequence identity or homology with respect to a neuregulin as referred to herein is the percentage of amino acid sequences of a neuregulin protein or fragment or derivative thereof that are identical with a specified sequence, after introducing any gaps necessary to achieve the maximum percent homology.

The neuregulin fragments and derivatives for use in the methods of the invention preferably exhibit good activity in standard neuroprotective assays such as

the *in vivo* cerebral ischemia assay of Example 1, which follows. That assay includes the following steps: a) continuous intraventricular infusion of the protein fragment or derivative or vehicle alone to test rats for three days prior to inducing focal ischemic infarcts in right lateral cerebral cortex; and b) twenty-four hours after inducing ischemic infarcts, infarct volume in each test animal is determined by image analysis. Preferably, a protein fragment or derivative of the invention provides at least about a 10% reduction in infarct volume relative to vehicle-treated animals, more preferably about a 20% reduction in infarct volume, still more preferably about a 25% reduction in infarct volume relative to vehicle-treated animals in such an assay. References herein to *in vivo* cerebral ischemia assay are intended to refer to an assay of the above steps a) and b), which are more fully described in Example 1 which follows.

As discussed above, neuregulin nucleic acid fragments and derivatives are also provided for use in the methods of the invention. Those fragments and derivatives typically are of a length sufficient to bind to a sequence of any of the nucleic acid sequences shown in Figures 1-15 of the drawings, including SEQ ID NOS:1, 3, 6, 7, 10, 11, 13, 16, 17, 20, 21, 24, 25, 28, 29, 31, 32, 34, 36, 37, 40, 42 and 43 under the following moderately stringent conditions (referred to herein as "normal stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.8M saline/0.08M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing once with that SSC buffer at 37°C.

Preferred neuregulin nucleic acid fragments and derivatives of the invention will bind to a sequence of any of the nucleic acid sequences shown in Figures 1-15 of the drawings, including SEQ ID NOS:1, 3, 6, 7, 10, 11, 13, 16, 17, 20, 21, 24, 25, 28, 29, 31, 32, 34, 36, 37, 40, 42 and 43 under the following highly stringent conditions (referred to herein as "high stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer at a temperature of 42°C and remaining bound when subject to washing twice with that SSC buffer at 42°C.

The neuregulin nucleic acid fragments and derivatives preferably should comprise at least 20 base pairs, more preferably at least about 50 base pairs, and still more preferably a nucleic acid fragment or derivative of the invention comprises at least about 100, 200, 300 or 400 base pairs. In some preferred embodiments, the

nucleic acid fragment or derivative is bound to some moiety which permits ready identification such as a radionucleotide, fluorescent or other chemical identifier.

Isolated neuregulin and peptide fragments or derivatives of the invention are preferably produced by recombinant methods, although suitable neuregulins also can
5 be isolated from various sources. See the procedures disclosed U.S. Patent 5,530,109; U.S. Patent 5,367,060; and PCT/US93/07491, incorporated herein by reference. A wide variety of molecular and biochemical methods are available for generating and expressing neuregulin; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor),
10 *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, neuregulin or fragments or derivatives thereof may be obtained by chemical synthesis, or more preferably by expression in bacteria such as *E coli* and eukaryotes such as yeast,
15 baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of neuregulin or fragment or derivative thereof. More particularly, a recombinant DNA molecule comprising a vector and a DNA segment encoding neuregulin, or a fragment or derivative thereof, can be constructed. Suitable vectors include e.g. baculovirus-derived vectors for expression in insect cells (see
20 Pennock et al., *Mol. Cell. Biol.*, 4:399-406 (1984)), T7-based expression vector for expression in bacteria (see Rosenberg et al., *Gene*, 56:125-135 (1987)) and the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.*, 263:3521-3527 (1988)). The DNA segment can be present in the vector operably linked to regulatory elements, e.g., a promoter (e.g., polyhedron, T7 or
25 metallothionein (Mt-I) promoters), or a leader sequence to provide for secretory expression of the polypeptide. The recombinant DNA molecule containing the DNA coding for a neuregulin or a fragment or derivative thereof can be introduced into appropriate host cells by known methods. Suitable host cells include e.g. prokaryotes such as *E. coli*, *Bacillus subtilis*, etc., and eukaryote such as animal cells and yeast
30 strains, e.g., *S. cerevisiae*. Mammalian cells may be preferred such as J558, NSO, SP2-O or CHO. In general, conventional culturing conditions can be employed. See Sambrook, *supra*. Stable transformed or transfected cell lines can then be selected.

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The expressed neuregulin or fragment or derivative thereof then can be isolated and purified by known methods. Typically the culture medium is centrifuged and the supernatant purified by affinity or immunoaffinity chromatography, e.g. Protein-A or Protein-G affinity chromatography or an immunoaffinity protocol comprising use of
5 monoclonal antibodies that bind neuregulins.

Neuregulin nucleic acids used in the methods of the invention are typically isolated, meaning the nucleic acids comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome and usually constitute at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by
10 weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given
15 fraction.

As discussed above, the present invention includes methods for treating and preventing certain neurological-related injuries and disorders, comprising the administration of an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, to a subject including a mammal, particularly
20 a human, in need of such treatment.

In particular, the invention provides methods for treatment and/or prophylaxis of nerve cell death (degeneration) resulting from hypoxia, hypoglycemia, brain or spinal cord ischemia, brain or spinal cord trauma, stroke, heart attack or drowning. Typical candidates for treatment include e.g. heart attack, stroke and/or persons
25 suffering from cardiac arrest neurological deficits, brain or spinal cord injury patients, patients undergoing major surgery such as heart surgery where brain ischemia is a potential complication and patients such as divers suffering from decompression sickness due to gas emboli in the blood stream. Candidates for treatment also will include those patients undergoing a surgical procedure involving extra-corporal
30 circulation such as e.g. a bypass procedure.

The invention also provides methods for treatment which comprise administration of a neuregulin or fragment or derivative thereof, or nucleic acid

encoding same, to a patient that is undergoing surgery or other procedure where brain or spinal cord ischemia is a potential risk. For example, carotid endarterectomy is a surgical procedure employed to correct atherosclerosis of the carotid arteries. Major risks associated with the procedure include intraoperative embolization and the danger of hypertension in the brain following increased cerebral blood flow, which may result in aneurysm or hemorrhage. Thus, an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, could be administered pre-operatively or peri-operatively to reduce such risks associated with carotid endarterectomy, or other post-surgical neurological deficits.

10 The invention also is effective to promote and enhance recovery from acute nerve cell death and neurological conditions. Thus, for example, a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, could be administered to promote repair, remodeling or reprogramming to a patient that has suffered from stroke or other neuronal injury, suitably for an extended period as discussed above. A therapeutic agent of the invention also could be administered post-operatively to promote recovery from any neurological deficits that may have occurred to a patient that has undergone surgery.

15 The invention further includes methods for prophylaxis against neurological deficits resulting from e.g. coronary artery bypass graft surgery and aortic valve replacement surgery, or other procedure involving extra-corporal circulation. Those methods will comprise administering to a patient undergoing such surgical procedures an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, typically either pre-operatively or peri-operatively.

20 The invention also provides methods for prophylaxis and treatment against neurological injury for patients undergoing myocardial infarction, a procedure that can result in ischemic insult to the patient. Such methods will comprise administering to a patient undergoing such surgical procedure an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, typically either pre-operatively or peri-operatively.

25 Also provided are methods for treating or preventing neuropathic pain such as may be experienced by cancer patients, persons having diabetes, amputees and other persons who may experience neuropathic pain. These methods for treatment comprise

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administration of an effective amount of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, to a patient in need of such treatment.

The invention also provides methods for treatment and prophylaxis against retinal ischemia or degeneration and resulting visual loss. For example, a neuregulin or fragment or derivative thereof, can be administered parenterally or by other
5 procedure as described herein to a subject suffering from or susceptible to ischemic insult that may adversely affect retinal function, e.g., significantly elevated intraocular pressures, diseases such as retinal artery or vein occlusion, diabetes or other ischemic ocular-related diseases. Post-ischemic administration also may limit retinal damage.

10 The invention also includes methods for treating and prophylaxis against decreased blood flow or nutrient supply to retinal tissue or optic nerve, or treatment or prophylaxis against retinal trauma or optic nerve injury. Subjects for treatment according to such therapeutic methods of the invention may be suffering or susceptible to retinal ischemia that is associated with atherosclerosis, venous capillary
15 insufficiency, obstructive arterial or venous retinopathies, senile macular degeneration, cystoid macular edema or glaucoma, or the retinal ischemia may be associated with a tumor or injury to the mammal. Intravitreal injection also may be a preferred administration route to provide more direct treatment to the ischemic retina.

The invention further provides a method of treating Korsakoff's disease, a
20 chronic alcoholism-induced condition, comprising administering to a subject including a mammal, particularly a human, an effective amount of a neuregulin or fragment or derivative thereof, in an amount effective to treat the disease.

Compounds of the invention are anticipated to have utility for the attenuation of cell loss, hemorrhages and/or amino acid changes associated with Korsakoff's disease.

25 The invention further includes methods for treating a person suffering from or susceptible to epilepsy, emesis, narcotic withdrawal symptoms and age-dependent dementia, comprising administering to a subject including a mammal, particularly a human, an effective amount of a neuregulin or fragment or derivative thereof, in an amount effective to treat the condition.

30 It will be appreciated that in some instances a neuregulin or a fragment or derivative thereof will be preferably administered to a subject rather than a neuregulin nucleic acid, particularly where a patient is suffering from or susceptible to an acute

neurological injury that demands immediate therapy. For example, administration of a neuregulin polypeptide may be preferred to a patient suffering from stroke, heart attack, traumatic brain injury and the like where it is desired to deliver the active therapeutic as quickly as possible.

5 In the therapeutic methods of the invention, neuregulin peptides and nucleic acids may be suitably administered to a subject such as a mammal, particularly a human, by any of a number of routes including parenteral (including subcutaneous, intramuscular, intravenous and intradermal), oral, rectal, nasal, vaginal and optical (including buccal and sublingual) administration. A neuregulin protein or nucleic acid
10 or fragment or derivative thereof may be administered to a subject alone or as part of a pharmaceutical composition, comprising the peptide or nucleic acid together with one or more acceptable carriers and optionally other therapeutic ingredients. The carriers should be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

15 Nucleic acids encoding a neuregulin or a neuregulin fragment or derivative can be administered to a patient by generally known gene therapy procedures. See, for example, WO 90/11092 and WO 93/00051. Thus, for instance, the nucleic acids may be introduced into target cells by any method which will result in the uptake and expression of the nucleic acid by the target cells. These methods can include vectors,
20 liposomes, naked DNA, adjuvant-assisted DNA, catheters, etc. Preferably, the administered nucleic acid codes for an appropriate secretory sequence to promote expression upon administration. Suitable vectors for administering a nucleic acid in accordance with the invention include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and
25 a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

30 Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox

vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem*, 64:487 (1995); F. Lim et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); A.I. Geller et al., *Proc Natl. Acad. Sci. U.S.A.*:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*, 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet.*, 3:219 (1993); Yang et al., *J. Virol.*, 69:2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cell cytoplasm. Avipox virus vectors result in only a short-term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the specific condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, $\text{Ca}_3(\text{PO}_4)_2$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, and viral vectors.

A vector can be employed to target essentially any desired target cell. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Additionally, the particles can be delivered by intracerebroventricular (icv) infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell (Bobo et al., *Proc. Natl. Acad. Sci. USA*, 91:2076-2080 (1994); Morrison et al., *Am. J. Physiol.*, 266:292-305 (1994)). Other methods that can be used include catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, and oral or other known routes of administration.

Parenteral formulations for administration of a neuregulin or a fragment or derivative thereof may be in the form of liquid solutions or suspensions; for oral

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administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences". Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for a neuregulin or fragments or derivatives thereof include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The concentration of a neuregulin or a fragment or derivative thereof, or nucleic acid encoding such polypeptides, administered to a particular subject will vary depending upon a number of issues, including the condition being treated, the mode and site of administration, the age, weight sex and general health of the subject, and other such factors that are recognized by those skilled in the art. Optimal administration rates for a given protocol of administration can be readily determined by those skilled in the art.

All documents mentioned herein are incorporated herein by reference in their entirety. The invention is further illustrated by the following non-limiting Examples. Example 1 -- In vivo neuroprotection assay

Neuregulins and neuregulin fragments and derivatives can be assessed for neuroprotective efficacy pursuant to the following assay.

Mature male Long-Evans rats (Charles River, 250-350g) are allowed food and water *ad libitum*. Animals are anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic head holder (David Kopf Instruments, Tujunga, CA). The

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dorsal surface of the skull is exposed by midline incision, and a small burr hole (2 mm diameter) is drilled over the right lateral ventricle, 1.6 mm lateral and 0.9 mm posterior to bregma. A stainless steel cannula (I.D. 0.020", O.D. 0.028", 2 cm long) is then inserted stereotaxically into the ventricle to a depth of 4.4 mm beneath the surface of the skull. The tubing is suitably bent at a 90° angle 1-1.6 cm from its tip and connected to polyethylene tubing (I.D. 0.76 mm, O.D. 1.22 mm, 10 cm long) that is connected (by glue) to a mini-osmotic pump (Alzet 1007D, 100 µl fill volume, pump rate = 0.5 µl/hr; Alza Corp., Palo Alto, CA) implanted subcutaneously in the back. The cannula can be suitably fixed to the skull by orthodontic resin (L.D. Culk Co., Milford, DE) bonded to two small machine screws (1/8" stainless steel slotted) inserted in the skull. The pump, tubing, and cannula are primed before insertion with infusate solutions; a 3-0 nylon suture is inserted into the cannula during implantation to prevent obstruction by brain tissue. The wound is closed with 3-0 silk suture and cefazolin (10 mg, i.m.) is administered. After surgery animals are suitably kept in individual cages and fed soft food.

Pumps are filled with vehicle alone (containing 127 mM NaCl, 2.6 mM KCl, 1.2 mM CaCl₂, 0.9 mM MgCl₂, 4.14 mM HEPES, 3 mM glycerin, 0.001% bovine serum albumin [BSA], and 0.01% fast green), or vehicle neuregulin or fragment or derivative thereof (100 µgm/ml). Heparin can be suitably used at relatively low doses, e.g. about 0.8 units/kg/day which is approximately 250-500 times less than a standard anticoagulant dose.

Three days after cannula implantation, animals are reanesthetized with 2% halothane and given atropine (0.15 mg/kg, i.p.). Animals are then intubated and connected to a ventilator (SAR-830; CWE Inc., Ardmore, PA) delivering 1% halothane/70% nitrous oxide in oxygen. The right femoral artery and vein are cannulated for monitoring of mean arterial blood pressure (MABP; Gould RS3200 Blood Pressure Monitor, Gould Inc., Valley View, OH), and blood sampling. Animals are then paralyzed with pancuronium bromide (0.5 mg/kg, i.v.). Arterial blood gasses (Corning 178 Blood Gas Analyzer, Ciba Corning Diagnostic Corp., Medford, MA), blood glucose (Accu-Check Blood Glucose Analyzer, Boehringer Mannheim, Indianapolis, IN), and hematocrit are measured at least twice during surgery and the immediate post-operative period. The stroke volume and rate of the

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ventilator are adjusted to maintain PaO_2 between 100-200 mm Hg and PaCO_2 between 30-40 mm Hg. Core body temperature may be monitored by rectal thermocouple (e.g. Model 73ATA, Yellow Springs Instrument Co., Yellow Springs, OH) and maintained between 36-37°C with a homeothermic blanket control unit (Harvard Bioscience, South Natick, MA).

Focal ischemic infarcts are made in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA) by the method of Chen, et al., *Stroke*, 17:738-743 (1986). Both common carotid arteries are exposed by midline anterior cervical incision. The animal is placed in a lateral position and a 1 cm skin incision is then made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle is retracted, and a small (3 mm diameter) craniectomy is made at the junction of the zygoma and squamosal bone using a dental drill cooled with saline. Using a dissecting microscope, the dura can be opened with fine forceps, and the right MCA can be ligated with two 10-0 monofilament nylon ties just above the rhinal fissure and transected between the ties. Both common carotid arteries then can be occluded by microaneurysm clips for 45 minutes. After removal of the clips, return of flow is visualized in the arteries. Anesthesia is maintained for 15 minutes, and animals are returned to individual cages and fed soft food after surgery.

Twenty four hours after cerebral infarction, animals are again weighed, and then sacrificed by rapid decapitation. Brains are removed, inspected visually for the anatomy of the middle cerebral artery as well as for signs of hemorrhage or infection, immersed in cold saline for 10 minutes, and sectioned into six standard coronal slices (each 2 mm thick) using a rodent brain matrix slicer (Systems, Warren, MI). Brains are also examined visually for the presence of dye (fast green) in the cerebral ventricles. Slices are placed in the vital dye 2,3,5-triphenyl tetrazolium chloride (TTC, 2%; Chemical Dynamics Co., NH) at 37°C in the dark for 30 minutes, followed by 10% formalin at room temperature overnight. The outline of right and left cerebral hemispheres as well as that of infarcted tissue, clearly visualizable by lack of TTC staining (Chen et al., *Stroke*, 17:738-743 (1986)), is outlined on the posterior surface of each slice using an image analyzer (MTI videocamera and Sony video monitor connected to a Bioquant IV Image Analysis System run on an EVEREX computer). Infarct volume is calculated as the sum of infarcted area per slice multiplied by slice

thickness. Both the surgeon and image analyzer operator are blinded to the treatment given each animal.

Volumes of infarcts among vehicle vs. neuregulin-treated animals can be compared by unpaired, two-tailed t-tests for each experiment, and by two-way analysis of variance (ANOVA; Exp. X Treatment) for combined data. A subsequent slice-by-slice analysis of infarct area among pooled neuregulin- vs. vehicle-treated animals is suitably done by repeated measures two-way ANOVA (Treatment X Slice). Other anatomical and physiological measurements are compared among GDF-1- vs. vehicle-treated animals by unpaired, two-tailed t-tests using the Bonferroni correction for multiple pairwise comparisons.

Example 2 -- In vivo behavioral assays

For behavioral outcome studies, such as to assess recovery, repair and remodeling promoted by administration of a neuregulin or fragment or derivative thereof, or nucleic acid encoding same, a number of assays can be employed such as those described in G. Sinson et al., *J Neurochem*, 65(5):2209-2214 (1995); T.K. McIntosh et al., *Neuroscience*, 28:233-244 (1989); and T.K. McIntosh et al., *J Neurotrauma*, 10:373-384 (1993).

Briefly, one suitable behavioral assay as described in G. Sinson et al., *supra*, entails that test animals (male Sprague-Dawley rats) receive preinjury training in a Morris Water Maze, a circular tank 1 m in diameter that is filled with 18°C water. The water surface is made opaque with a covering of Styrofoam pieces. During training of the animals a submerged platform is present in the maze. Each test animal undergoes 20 training trials over a two day period during which they learn to locate the platform using external visual cues. Immediately following the last training trial, animals are anesthetized and subjected to a lateral (parasagittal) fluid-percussion (FP) brain injury. Briefly, a 5-mm craniectomy is performed over the left parietal cortex, midway between lamda and bregma. A hollow Leur-loc fitting is cemented to the craniectomy site. The injury is delivered after attaching the FP device. The injury should be of moderate severity (2.1-2.3 atm). After injury, the Leur-loc is removed, and the skin is sutured. Normothermia is maintained with warming pads until the animals begin to ambulate.

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At 72 hours, 1 week or 2 weeks after injury, animals are assessed for their ability to remember the learned task of locating the platform in the MWM. For this evaluation the platform is removed from the maze, and the animal's swimming pattern is suitably recorded with a computerized video system for 1 minute. The maze is
5 separated in zones that are weighed according to the proximity to the platform's location. A memory score is generated by multiplying the weighted numbers by the time the animal spends in each zone and then adding the products.

Animals surviving for 1 or 2 weeks also can undergo evaluation of neurologic motor function. Briefly, one suitable assay provides that animals are scored from 0
10 (severely impaired) to 4 (normal) for each of the following: (1) left and (2) right forelimb during suspension by the tail; (3) left and (4) right hindlimb flexion when the forelimbs remain on a surface and the hindlimbs are lifted up and back by the tail; the ability to resist lateral pulsion to the (5) left and (6) right; and the ability to stand on an inclined plane in the (7) left, (8) right, and (9) vertical positions. Scores are
15 combined for each of the tests (1) through (9). The observer for the tests should be blinded to the animal's previous treatment.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements
20 within the spirit and scope of the invention.